# A Simple Procedure for the Preliminary Identification of Aerobic Gram Negative Intestinal Bacteria with Special Reference to the Enterobacteriaceae

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ABSTRACT

**A useful procedure is described for rapid identification of some Gram negative bacteria during quantitative studies of the intestinal flora from various species. The methods used are based on primary isolation and differentiation of the bacteria by the use of MacConkey agar and the subsequent differentiation according to a definite procedure using the replica plating technique.**

RÉSUMÉ

**Les auteurs décrivent un procédé utile pour l'identification rapide de certaines bactéries Gram-, dans le cadre d'études quantitatives de la flore intestinale, chez différentes espèces. Les méthodes utilisées sont basées sur l'isolement et l'identification primaires des microbes, en utilisant la gélose MacConkey, ainsi que sur la différenciation ultérieure selon un procédé bien déterminé qui consiste dans l'emploi de la méthode des répliques.**

## INTRODUCTION

Various biochemical tests and procedures already established for the identification of the aerobic Gram negative bacteria especially the *Enterobacteriaceae* were summarized by Traub *et al* (6). These tests and procedures have been widely used in the isolation and enumeration of bacteria from various sources. However, some of the tests employed require a large quantity of culture media and a prolonged incubation, thus delaying results considerably. Moreover, some laboratories do not have the facilities and the staff required to follow the procedures for such quantitative studies. The present paper describes a method which allows the rapid quantitative study of the aerobic Gram negative bacteria from various sources in any laboratory supplied with a minimum of facilities and personnel. This method combines some specific biochemical tests for the identification of aerobic Gram negative, lactose fermenting (LF) and non-lactose fermenting (NLF) bacteria with the replica plating method (5).

## MATERIALS AND METHODS

A total of 28 strains representing 14 genera (*Escherichia, Klebsiella, Enterobacter, Pseudomonas, Alcaligenes, Aeromonas,*

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Submitted March 10, 1972.

*Proteus, Providencia, Citrobacter, Shigella, Salmonella, Arizona, Serratia, Edwardsiella*) were used. All the strains were restreaked on MacConkey agar and utilized after a second subculture. The tests or substrates used and the corresponding media are summarized in Table I. All the media were prepared from commercially available (Difco[1] and BBL[2]) dehydrated stocks. The excess wetness from the media was eliminated by overnight incubation of the plates at 37°C to reduce spreading during transfer operation.

An identification procedure was devised and is illustrated in Fig. 1. Criteria for identification of cultures were taken from the various publications of Ewing and coworkers, as summarized by Ewing (2), from Hendrie and Shevan (3) and Ewing and Johnson (1). The choice of the differential tests used was based on the fact that they gave a positive or a negative result in 90-100% of the strains studied.

[1]Difco Laboratories, Detroit, Michigan 4820, U.S.A.

[2]Baltimore Biological Laboratory, Division of Bioquest, Division of Becton Dickinson and Co., Cockeysville, Maryland 21030, U.S.A.

**TABLE I. Different Tests Used in the Identification of Selected Gram Negative Bacteria**

| Test or Substrate | Medium |
|---|---|
| (1) Lactose | MacConkey Agar |
| (2) Rhamnose | MacConkey Agar base + 0.5% Rhamnose |
| (3) Glucose[c] | Seller's Differential Agar |
| (4) Glucose | MacConkey Agar base + 0.5% glucose |
| (5) Citrate | Simmons' citrate |
| (6) Malonate | Malonate base + 0.03% bactopeptone |
| (7) Urease[a, c] | Urea Agar Base |
| (8) P.P.A.[b, c] | Phenyl-alanine Agar |
| (9) Lysine decarboxylase[c] | Lysine Decarboxylase Broth + 0.1% lysine + 2.0% Ionagar (Oxoid) |
| (10) Cytochrome oxidase[b] | MacConkey Agar |

[a]The phenyl pyruvic acid production is shown by flooding the plates with the following reagent: ammonium sulfate 2.0 g; sulphuric acid (10%), 1.0 ml; half-saturated ferric alun, 5.0 ml. After one or two minutes an intensive blue color developing in the colonies indicates a positive reaction

[b]Cytochrome oxidase activity is shown by flooding the plates with a freshly prepared 1.0% solution of tetramethy-p-phenylenediamine. Oxidase positive colonies develop a pink color, which successively becomes maroon, dark red, black in ten to 30 minutes (4)

[c]Incubation under anaerobic conditions

To test the validity of this procedure, colonies of each of the 28 strains were inoculated on a MacConkey agar plate and the position of each strain was recorded. After incubation at 37°C overnight the lactose fermenting (LF) and non-lactose fermenting (NLF) colonies were reinoculated separately on MacConkey agar plates. After incubation, the reisolated colonies were transferred to the corresponding differential media, by the replica plating method. The different plates were then incubated at 37°C, under the required aerobic or anaerobic conditions. Observations of replicated colonies were made at various time intervals to determine the interval incubation time for each test or substrate used. Anaerobic conditions were achieved in Gas-Pack anaerobic jars (BBL).

## RESULTS

The optimum incubation time for urease and lysine decarboxylase is four to five hours, while for the other tests it is eight to 20 hours. Once differentiated on MacConkey agar plates into lactose-fermenting (LF) and non-lactose fermenting (NLF) groups, colonies of each group may be further differentiated into the corresponding genera as follows:

### LF GROUPS

This group includes *Escherichia, Klebsiella* and *Enterobacter*. *Klebsiella* and *Enterobacter* can utilize citrate as their unique source of carbon, while *Escherichia* cannot. Growth surrounded by a blue halo developing on Simmons' citrate indicates the presence of *Klebsiella* or *Enterobacter*. Our attempts to carry out the methyl-red and Voges-Proskauer tests on solid media proved unsuccessful, and we have further differentiated *Klebsiella* and *Enterobacter* by their morphological features on MacConkey agar base supplemented with 0.5% glucose. Small red, yellow centered colonies are produced by *Enterobacter*, while *Klebsiella* gives rise to mucoid yellow colonies with red border.

### NLF GROUPS

This group includes all the remaining genera, which can be primarily subdivided

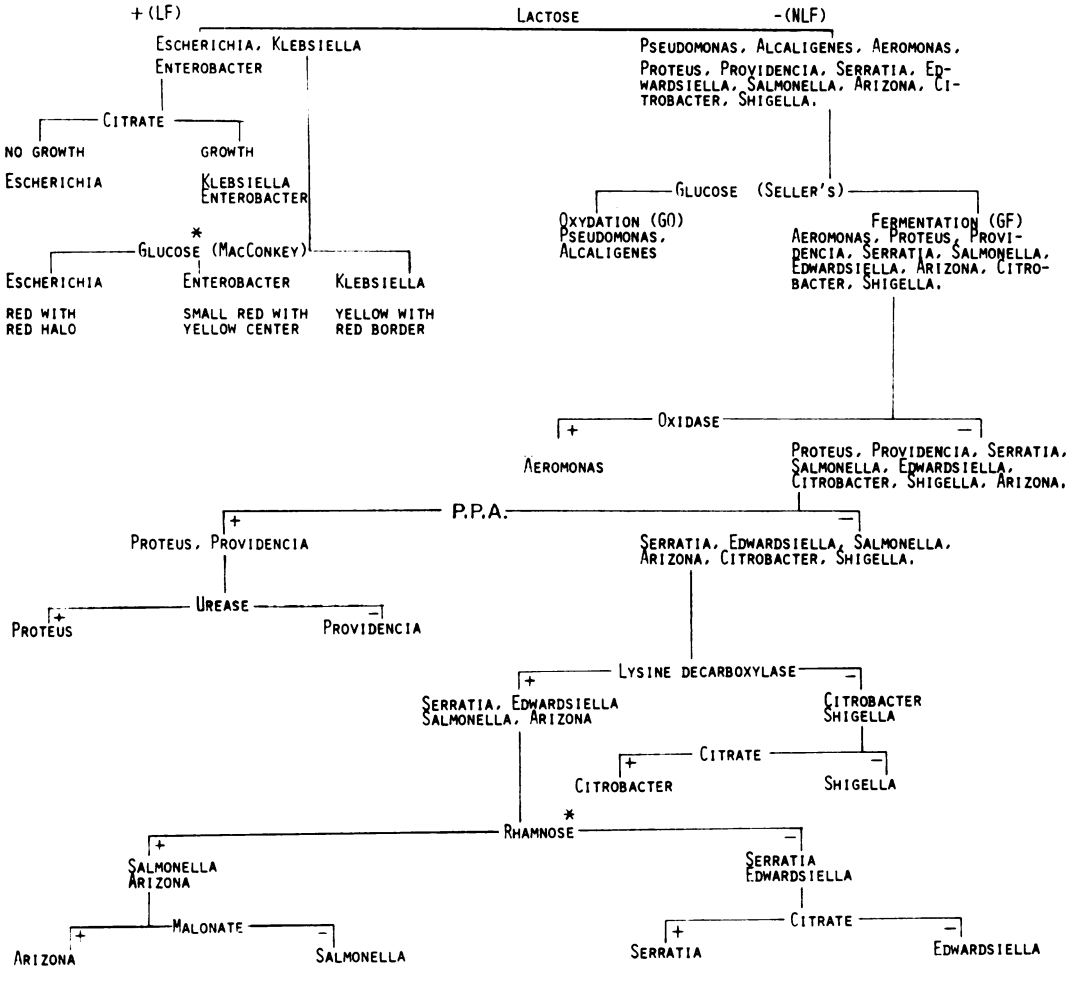
into a glucose oxidizing (GO) group and a glucose fermenting (GF) group using Seller's differential agar.

A— GO GROUP

This group includes *Pseudomonas* and *Alcaligenes*. After 24 hours incubation at 37°C under anaerobic conditions, the following features are observed on Seller's differential agar. Colonies of *Pseudomonas* are small greenish-blue in the case of *Ps. fluorescens*, while those of *Ps. aeruginosa* are large, blue and surrounded by a faint blue halo. *Alcaligenes faecalis* gives rise to violet-blue, white-centered colonies surrounded by a deep-blue halo.

B— GF GROUP

Members of this group incubated in the same conditions as above acidify Seller's differential agar giving rise to yellow colonies. To differentiate *Aeromonas* from the other members of this group, use is made of the fact that only *Aeromonas* is cytochrome oxidase positive while *Enterobacteriaceae* are not (1). Thus on MacConkey




+: INDICATES A POSITIVE REACTION OR THE SUBSTRATE UTILISATION BY THE BACTERIA.

-: INDICATES A NEGATIVE REACTION OR THE NON-UTILISATION OF THE SUBSTRATE BY THE BACTERIA.

* in Mac Conkey agar base.

**Fig. 1. Identification procedure for Gram negative bacteria.**

agar, flooded with Kovac's reagent, only *Aeromonas* colonies will appear pink or maroon. On phenyl-alanine agar only *Proteus* and *Providencia* are phenyl pyruvic acid producers. *Proteus* is further differentiated from *Providencia* by its ability to hydrolyse urea on urea agar base. Among the remaining genera only *Citrobacter* and *Shigella* are lysine decarboxylase negative thus giving rise, on lysine decarboxylase agar, to yellow colonies among the pink colonies of the lysine decarboxylase positive genera. To differentiate *Citrobacter* from *Shigella,* use is made of the fact that on Simmons' citrate only *Citrobacter* can utilise citrate as a carbon source while *Shigella* cannot. The four genera left, *Salmonella, Arizona, Serratia* and *Edwardsiella* are best differentiated by the ability of *Salmonella* and *Arizona.* to ferment rhamnose. Further differentiation between these two genera is achieved using malonate base enriched with 0.03% bactopeptone to promote rapid growth; under these conditions, growth of Arizona organisms was observed as early as eight hours after inoculation whereas no growth of the Salmonella organisms were detected even after incubation periods of up to three days. Finally, *Serratia* is well differentiated from *Edwardsiella* by its ability to utilize citrate as a carbon source. The strains of *Serratia* utilized throughout this study were blood-red pigmented and could be easily differentiated on the starting MacConkey agar plates. However, the occurrence of non-pigmented strains being possible during any quantitative study, the complete differentiation procedure was carried out.

## DISCUSSION

A replica plating method has been already devised by Wiseman and Sarles (7) for screening intestinal coliform bacteria in chickens. While their method was restricted to only three coliform genera, the method described here includes all the *Enterobacteriaceae* genera and some of the *Pseudomonadaceae* genera. The use of our method allows the rapid identification of numerous isolates from various sources with great economy of time, labour and materials. This method was applied in our laboratory and proved to be useful for the identification of most aerobic Gram negative bacteria from the intestinal tract of fifteen pigs (Elazhary *et al,* in preparation). Moreover, we were able, starting from MacConkey plates with confluent growth of lactose fermenters and by replica plating on Simmons' citrate, to isolate *Klebsiella* or *Enterobacter* organisms which were overgrown by *E. coli.* This valuable feature renders this technique a powerful tool for the isolation, enumeration and identification of most of the aerobic Gram negative bacteria.

## ACKNOWLEDGMENTS

We wish to thank the Department of Immunology and Microbiology of the Faculty of Medicine of Montreal University, as well as the Faculty of Medicine of Laval University, from which some of the strains were obtained.

The assistance of technicians in our microbiology laboratory is also gratefully acknowledged.

This research work was supported by grant no. MMV70352 from "Le Conseil des Recherches Agricoles, Ministère de l'Agriculture et de la Colonisation du Québec".

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